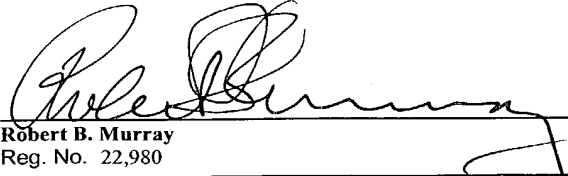


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|---|--|--|--|--|
| FORM PTO-1390<br>(REV 5-93)   |  | U.S. DEPARTMENT OF COMMERCE<br>PATENT AND TRADEMARK OFFICE |  | ATTORNEY DOCKET NO.<br>100564-00111                                      |
| TRANSMITTAL LETTER TO THE UNITED STATES<br>DESIGNATED/ELECTED OFFICE (DO/EO/US)<br>CONCERNING A FILING UNDER 35 U.S.C. 371  |  |  |  | DATE: March 29, 2002   |
|   |  |  |  | U.S. APPLN. NO.<br>(IF KNOWN, SEE 37 C.F.R. 1.5)<br>New <b>107089521</b> |
| INTERNATIONAL APPLICATION NO.<br>PCT/EP00/09588   |  | INTERNATIONAL FILING DATE<br>September 29, 2000            |  | PRIORITY DATE CLAIMED<br>September 29, 1999                              |
| TITLE OF INVENTION: USE OF GANGLIOSIDES AND OTHER SUBSTANCES FOR MODULATING SPHINGOLIPID-CHOLESTEROL MICRODOMAINS   |  |  |  |  |
| APPLICANT(S) FOR DO/EO/US: Mikael SIMONS, Tim FRIEDRICHSON, Kai SIMONS and Teymuraz KURZCHALIA  |  |  |  |  |
| <ol style="list-style-type: none"> <li>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.<br/>(THE BASIC FILING FEE IS ATTACHED)</li> <li>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures [35 U.S.C. 371(f)] at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</li> <li>4. <input checked="" type="checkbox"/> A proper demand for International Preliminary Amendment was made by the 19th month from the earliest claimed priority date.</li> <li>5. <input checked="" type="checkbox"/> A copy of the International Application as filed [35 U.S.C. 371(c)(2)]               <ol style="list-style-type: none"> <li>a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> has been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li> <li>6. <input type="checkbox"/> A translation of the International Application into English [35 U.S.C. 371(c)(2)].</li> <li>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 [35 U.S.C. 371(c)(3)]               <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> have been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 [35 U.S.C. 371(c)(3)].</li> <li>9. <input type="checkbox"/> An oath or declaration of the inventor(s) [35 U.S.C. 371(c)(4)].</li> <li>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 [35 U.S.C. 371(c)(5)].</li> </ol> <p>Items 11 - 16 below concern other document(s) or information included:</p> <ol style="list-style-type: none"> <li>11. <input type="checkbox"/> An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.</li> <li>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.</li> <li>13. <input type="checkbox"/> A FIRST preliminary amendment.<br/><input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</li> <li>14. <input type="checkbox"/> A substitute specification.</li> <li>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</li> <li>16. <input checked="" type="checkbox"/> Drawings (<input checked="" type="checkbox"/> FIGS. 1A-9 7 sheets)</li> </ol> |  |  |  |  |

JC13 Rec'd PCT/PTO 29 MAR 2002

|  |              |   |            |  |              |
|--|--------------|---|------------|--|--------------|
| U.S. APPL. NO. (IF KNOWN)<br>SEE 37 C.F.R. 1.50) New<br><b>10/089521</b>   |              | INTERNATIONAL APPLICATION<br>NO. PCT/EP00/09588 |            | ATTORNEY DOCKET NO. 100564-00111<br>DATE: March 29, 2002   |              |
| 17. <input checked="" type="checkbox"/> The following fees are submitted:<br><b>Basic National Fee [37 C.F.R. 1.492(a)(1)-(5)]:</b><br>Search Report has been prepared by the EPO or JPO.....\$890.00<br>International preliminary examination fee paid to USPTO<br>(37 C.F.R. 1.482).....\$710.00<br>No international preliminary examination fee paid to USPTO<br>(37 C.F.R. 1.482) but international search fee paid to USPTO<br>[37 C.F.R. 1.445(a)(2)].....\$740.00<br>Neither international preliminary examination fee<br>(37 C.F.R. 1.482) or international search fee<br>[37 C.F.R. 1.445(a)(2)] paid to USPTO.....\$1,040.00<br>International preliminary examination fee paid to USPTO<br>(37 C.F.R. 1.482) and all claims satisfied provisions of<br>PCT Article 33(2)-(4).....\$ 100.00 |              |   |            | CALCULATIONS   | PTO USE ONLY |
|  |              |   |            |  |              |
| <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>  |              |   |            | \$ 890.00  |              |
| Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date [37 C.F.R. 1.492(e)].   |              |   |            | \$   |              |
| Claims   | Number Filed | Number Extra                                    | Rate       |  |              |
| Total Claims   | - 20 =       |   | X \$ 18.00 | \$   |              |
| Independent Claims   | - 3 =        |   | X \$ 84.00 | \$   |              |
| Multiple dependent claim(s) (if applicable)  |              |   | + \$280.00 | \$   |              |
| <b>TOTAL OF ABOVE CALCULATIONS =</b>   |              |   |            | \$ 890.00  |              |
| Reduction by one-half for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 C.F.R. 1.9, 1.27, 1.28).   |              |   |            | \$   |              |
| <b>SUBTOTAL =</b>  |              |   |            | \$ 890.00  |              |
| Processing fee of \$130.00 for furnishing the English translation later the <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date [37 C.F.R. 1.492(f)].   |              |   |            | \$   |              |
| <b>TOTAL NATIONAL FEE =</b>  |              |   |            | \$ 890.00  |              |
| Fee for recording the enclosed assignment [37 C.F.R. 1.21(h)]. The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). \$40.00 per property   |              |   |            | \$   |              |
| <b>TOTAL FEES ENCLOSED =</b>   |              |   |            | \$ 890.00  |              |
|  |              |   |            | Amount to be refunded  | \$           |
|  |              |   |            | Charged  | \$           |
| a. <input checked="" type="checkbox"/> A check in the amount of \$890.00 to cover the above fees is enclosed.<br>b. <input type="checkbox"/> Please charge my Deposit Account No. 01-2300 in the amount of \$                      to cover the above fee.<br>A duplicate copy of this sheet is enclosed.<br>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 01-2300.   |              |   |            |  |              |
| NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive [37 C.F.R. 1.137(a) or (b)] must be filed and granted to restore the application to pending status.  |              |   |            |  |              |
| SEND ALL CORRESPONDENCE TO:<br><b>Arent Fox Kintner Plotkin &amp; Kahn</b><br><b>1050 Connecticut Avenue, N.W.</b><br><b>Suite 400</b><br><b>Washington, D.C. 20036-5339</b><br><b>Tel: (202) 857-6000 Fax: (202) 638-4810</b><br><b>RBM/ars</b>   |              |   |            |  |              |
|  |              |   |            | <br><b>Robert B. Murray</b><br>Reg. No. 22,980 |              |

1008952102  
10/08952102  
23 Aug 2002

**PATENT APPLICATION**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:

SIMONS et al.

Application No.: 10/089,521

Examiner: Unknown

Filed: March 29, 2002

Attorney Dkt. No.: 100564-00111

For: USE OF GANGLIOSIDES AND OTHER SUBSTANCES FOR MODULATING  
SPHINGOLIPID-CHOLESTEROL MICRODOMAINS

**PRELIMINARY AMENDMENT**

Commissioner for Patents  
Washington, D.C. 20231

August 23, 2002

Sir:

Prior to initial examination of the application, please amend the above-identified application as follows:

**IN THE CLAIMS:**

Please amend claims 3, 5, 6, 7, 8, 9, 10, 11, 12, and 13 as follows:

3. (Amended) The use as claimed in claim 1, characterized in that the agent influences the location of proteins on the sphingolipid-cholesterol microdomains.

5. (Amended) The use as claimed in claim 3, characterized in that the agent acts on glycosylphosphatidylinositol anchor proteins, kinases of the Src family, influenza virus hemagglutinin and other viral proteins and/or caveolin-1, 2 or 3 in the sphingolipid-cholesterol microdomain.

6. (Amended) The use as claimed in claim 1, characterized in that the agent brings about a disassembly of the protein clusters.

7. (Amended) The use as claimed in claim 1, characterized in that the ganglioside is selected from bovine brain gangliosides, GM<sub>1</sub>, GD1a, GD1b, GD3, GM2, GM3, GQ1a, GQ1b, and/or globosides and their derivatives, in particular unsaturated sphingosines or ceramides containing unsaturated or short fatty acids.

8. (Amended) The use as claimed in claim 1, characterized in that cholesterol derivatives, in particular cholesterol sulfate, are employed.

9. (Amended) The use as claimed in claim 1, characterized in that the modulation of the sphingolipid cholesterol microdomains brings about a change in membrane transport, signal transmission and/or cell adhesion properties and/or in enzymic processes.

10. (Amended) The use as claimed in claim 1, characterized in that the modulation of the sphingolipid-cholesterol microdomains brings about a change in the proteolysis of the amyloid precursor protein of Alzheimer's disease or a modification in a prion protein.

11. (Amended) The use as claimed in claim 1, characterized in that the modulation of the sphingolipid-cholesterol microdomains prevents the phagocytosis of bacteria and parasites in mammalian cells.

12. (Amended) The use as claimed in claim 1, characterized in that the modulation of the sphingolipid-cholesterol microdomains prevents the uptake of viruses into mammalian cells and/or their transport and release.

13. (Amended) The use as claimed in claim 1, characterized in that the agent is employed as a therapeutic agent.

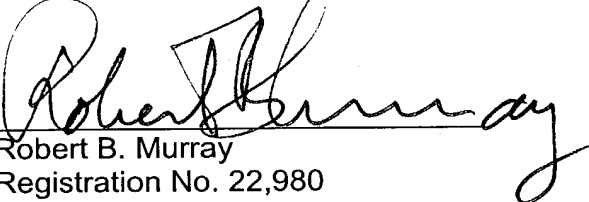
#### REMARKS

Claims 1-15 are pending in this application. By this Amendment, claims 3, 5, 6, 7, 8, 9, 10, 11, 12, and 13 are amended to remove multiple dependency. No new matter is contained in the amendments.

The application Declaration was filed on May 10, 2002.

Please charge any fee deficiency or credit any overpayment to Deposit Account No. 01-2300.

Respectfully submitted,



Robert B. Murray  
Registration No. 22,980

Customer No. 004372  
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## Patent claims

1. The use of gangliosides, ganglioside derivatives  
and/or cholesterol derivatives for preparing an  
agent for modulating sphingolipid-cholesterol  
microdomains.
2. The use as claimed in claim 1,  
characterized in that  
the agent influences the location of components  
and their function on the sphingolipid-cholesterol  
microdomains.
3. The use as claimed in claim 1 <sup>or 2</sup>,  
characterized in that  
the agent influences the location of proteins on  
the sphingolipid-cholesterol microdomains.
4. The use as claimed in claim 3,  
characterized in that  
the agent influences the location of anchor  
proteins, acylated proteins, Src kinases and/or  
cholesterol-anchored proteins and other raft  
proteins.
5. The use as claimed in claim 3 <sup>or 4</sup>,  
characterized in that  
the agent acts on glycosylphosphatidylinositol  
anchor proteins, kinases of the Src family,  
influenza virus hemagglutinin and other viral  
proteins and/or caveolin-1, 2 or 3 in the  
sphingolipid-cholesterol microdomain.
6. The use as claimed in <sup>claim 1</sup> one of the preceding claims,  
characterized in that  
the agent brings about a disassembly of the  
protein clusters.

7. The use as claimed in <sup>(claim 1)</sup> one of the preceding claims, characterized in that the ganglioside is selected from bovine brain gangliosides, GM<sub>1</sub>, GD1a, GD1b, GD3, GM2, GM3, 5 GQ1a, GQ1b and/or globosides and their derivatives, in particular unsaturated sphingosines or ceramides containing unsaturated or short fatty acids.
- 10 8. The use as claimed in <sup>(claim 1)</sup> one of the preceding claims, characterized in that cholesterol derivatives, in particular cholesterol sulfate, are employed.
- 15 9. The use as claimed in <sup>(claim 1)</sup> one of the preceding claims, characterized in that the modulation of the sphingolipid cholesterol microdomains brings about a change in membrane transport, signal transmission and/or cell 20 adhesion properties and/or in enzymic processes.
10. The use as claimed in <sup>(claim 1)</sup> one of the preceding claims, characterized in that the modulation of the sphingolipid-cholesterol 25 microdomains brings about a change in the proteolysis of the amyloid precursor protein of Alzheimer's disease or a modification in a prion protein.
- 30 11. The use as claimed in <sup>(claim 1)</sup> one of the preceding claims, characterized in that the modulation of the phingolipid-cholesterol microdomains prevents the phagocytosis of bacteria and parasites in mammalian cells.
- 35 12. The use as claimed in <sup>(claim 1)</sup> one of the preceding claims, characterized in that

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the modulation of the sphingolipid-cholesterol microdomains prevents the uptake of viruses into mammalian cells and/or their transport and release.

5

13. The use as claimed in <sup>(claim)</sup> [one of the preceding claims], characterized in that the agent is employed as a therapeutic agent.

10

14. The use of gangliosides, ganglioside derivatives and/or cholesterol derivatives for modulating sphingolipid-cholesterol microdomains.

15

15. A process for modulating sphingolipid-cholesterol microdomains, characterized in that gangliosides, ganglioside derivatives and/or cholesterol derivatives are administered to a patient at a dose of from 3 mg to 30 mg per kg per day.



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Use of gangliosides and other substances for modulating  
sphingolipid-cholesterol microdomains

Description

5

The invention relates to the use of gangliosides and other substances for preparing an agent for modulating sphingolipid-cholesterol microdomains.

- 10 Sphingolipid-cholesterol microdomains, which are also termed sphingolipid-cholesterol rafts, are lateral arrangements of specific lipids, in particular sphingolipids including gangliosides and cholesterol, and also proteins, in cell membranes (K. Simons et al.,  
15 Nature, Vol. 387 (1997), 569-572; T. Friedrichson et al., Nature, Vol. 394 (1998), 802-805). These microdomains are small and highly dynamic structures which are formed by attractive forces between sphingolipids and cholesterol. A variety of proteins  
20 are associated with the sphingolipid-cholesterol structures, including GPI-APs (glycosylphosphatidyl anchor proteins), kinases of the Src family, influenza virus hemagglutinin (HA) and caveolin-1. Most of the proteins which are associated with the sphingolipid-  
25 cholesterol rafts contain post-translational lipid modifications, with GPI-anchored proteins being anchored in the cell membrane by way of a lipid unit whereas kinases of the Src family, NO synthases (NOS), HA and caveolin are acylated. The proteins which are  
30 associated with the rafts are normally only poorly soluble in cold, nonionic detergents, such as Triton X-100, a property which is possibly to be attributed to their lipophilic character. Complexes which are insoluble in Triton have been defined, with  
35 regard to their function, as being rafts, which are specifically enriched at specific lipids, in particular sphingolipids, including gangliosides, and cholesterol, whereas other lipids, such as glycerophospholipids, are

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selectively lacking in the Triton extracts (Brown et al., Trends Cell Biol. 2 (1992), 338-343; Fiedler et al., Biochemistry 32 (1993), 6365-6373).

5 The existence of sphingolipid-cholesterol microdomains or rafts in living cells, in particular the existence of microdomains of GPI-anchored proteins in living cells, which domains can be influenced by administering cholesterol, has recently been demonstrated by means of  
10 a chemical crosslinking approach (Friedrichson et al., see above) and a fluorescence energy transfer method (Varma et al., Nature 394 (1998), 798-802). It has furthermore been postulated that, as platforms to which proteins can bind, the rafts play an important role in  
15 membrane transport and signal transmission properties.

An object of the invention was therefore to provide an agent for modulating sphingolipid-cholesterol microdomains (and in particular their constitution  
20 and/or properties) in order to be able to selectively exert an effect on the processes which are influenced by these microdomains.

According to the invention, this object is achieved by  
25 using gangliosides and other substances for preparing an agent for modulating sphingolipid-cholesterol microdomains.

Within the context of the present invention, it has  
30 been possible to establish that proteins which are associated with sphingolipid-cholesterol microdomains, in particular GPI-anchored proteins, are present in the microdomains in the form of crosslinked clusters on the surface of living cells. Adding gangliosides and other  
35 substances, such as cholesterol sulfate, inhibits the crosslinking of proteins which are associated with the rafts, in particular GPI-anchored proteins, and, in

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addition to this, increases their solubility in detergents.

The administration of gangliosides and other substances consequently leads to a change in the structural constitution of the microdomains, such that these gangliosides and other substances can be used for modulating sphingolipid-cholesterol microdomains and the processes which take place in these domains.

It is assumed that the administration of gangliosides and other substances displaces proteins, in particular GPI-anchored proteins, from the sphingolipid-cholesterol microdomains as a result of the attractive forces between the anchor group and the surrounding lipids being disrupted. The consequence of this is that, when gangliosides and other substances are added, the proteins which are present in the sphingolipid-cholesterol microdomains are leached out of their lipid environment and are no longer associated with the rafts.

As a consequence of this, the addition of exogenous gangliosides alters, for example, the solubility of GH-DAF (growth hormone (GH) which is linked to the GPI anchor of the DAF (decay accelerating factor)), a form of growth hormone which is glycosylphosphatidyl inositol-anchored, in detergents. It is known that GPI-anchored proteins are essentially insoluble in nonionic detergents (Brown et al., Cell 68 (1992), 533-544) whereas a decrease in the cholesterol content in cells increases the detergent solubility of GPI-APs (Cerneus et al., J. Biol. Chem. 268 (1993), 3150-3155; Hanada et al., J. Biol. Chem. 270 (1995), 6254-6260). This means that the forces of interaction between the lipid constituents and the proteins in the microdomains are greater than the solubilizing forces due to nonionic detergents. However, if the microdomains are altered or

destroyed by removing the constituent cholesterol, the forces of interaction between the lipids and the proteins are then diminished and the proteins can be leached out of the rafts by nonionic detergents.

5

Surprisingly, it has now been found that the opposite applies when gangliosides and other substances, which are preferably also a lipid constituent of the rafts, are added, i.e. that the addition of gangliosides to cells increases the detergent solubility of GH-DAF. This observation is unexpected since it would be assumed that an increase in the concentration of a lipid which is present in the sphingolipid-cholesterol microdomains would stabilize these domains. In fact, however, the addition of gangliosides disrupts the crosslinking of the proteins and increases the detergent solubility of GH-DAF. Gangliosides consequently bring about a modulation of the microdomains, in particular a displacement of proteins, in particular GPI-APs, from the rafts.

This is confirmed by the fact that gangliosides disrupt the copatching of HA and GH-DAF. The copatching of two microdomain components is a consequence of the coalescence of a common lipid microdomain.

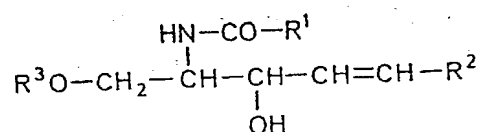
The gangliosides preferably modulate the microdomains specifically and reversibly, i.e. when the gangliosides and other substances are removed, the original, unaltered structure of the microdomains is obtained once again.

According to the invention, therefore, preference is given to using gangliosides to influence the location of proteins on sphingolipid-cholesterol microdomains, in particular the location of anchor proteins, acylated proteins, Src kinases and/or cholesterol-anchored or GPI-anchored proteins. Adding gangliosides alters the

arrangement of proteins which are present in sphingolipid-cholesterol microdomains, with this leading to a modulation and/or change in structure and/or constitution of the sphingolipid-cholesterol  
5 microdomains. Particular preference is given to the protein clusters being disassembled.

The gangliosides which are employed in accordance with the invention are preferably selected from bovine brain  
10 gangliosides, GM<sub>1</sub>, GD1a, GD1b, GD3, GM2, GM3, GQ1a, GQ1b and and/or globosides. Particular preference is given to using exogenous gangliosides and their derivatives, such as unsaturated sphingosine or ceramide containing short or unsaturated fatty acids  
15 which are already constituents of the sphingolipid-cholesterol microdomains to be treated. However, cholesterol sulfate and other cholesterol derivatives can also be employed.

20 The terms ganglioside and/or ganglioside derivatives, which are used herein, encompass, in particular, glycolipids which occur as structural elements of cell membranes in nerve tissue. The gangliosides and/or ganglioside derivatives preferably contain several  
25 monosaccharide units per molecule. Examples of suitable monosaccharide units which can be contained in the gangliosides and/or ganglioside derivatives are D-galactose, N-acetyl-D-galactosamine, D-glucose and N-acetylneuraminic acid. Particular preference is given  
30 to gangliosides which are derivatives of sphingosine (2-amino-4-octadecene-1,3-diol, sphing-4-enine), with, in particular, sugar residues being bonded on by way of the oxygen on the C-1 and a short (in particular C<sub>2</sub>-C<sub>18</sub>) fatty acid, which can be saturated or unsaturated,  
35 being bonded by way of the nitrogen on the C-2. Preference is furthermore given to the gangliosides which are a derivative of a ceramide. Ceramides are lipophilic amides of the formula



where  $R^1$  is a long-chain fatty acid residue, in particular a  $C_6$ - $C_{30}$ , more preferably a  $C_8$ - $C_{24}$ , fatty acid residue,  $R^2$  is a long-chain alkyl residue, in particular a  $C_6$ - $C_{30}$ , more preferably a  $C_8$ - $C_{24}$ , alkyl residue, and  $R^3$  is H. In the ceramide-containing gangliosides,  $R^3$  is preferably replaced with sugar residues (glycosides).

Consequently, ganglioside derivatives preferably comprise ceramide and its derivatives and related compounds including sphingosines and gangliosides and their derivatives, with it also being possible for one of the following functional groups to be substituted or added on the backbone chain, in particular the ceramide backbone chain: a halide atom, bonded to an alkyl, alkenyl, alkynyl or aryl radical, an alcohol group (primary, secondary or tertiary), an ether group, a carbonyl function (e.g. aldehyde or ketone), a carboxylic acid group, a carboxylic anhydride group, a carbamoyl group, a haloformyl group, a cyano group, an ester group, including a lactone group, a benzyl, phenyl, tolyl, tosyl or sulfonyl group, an amino group (primary, secondary or tertiary), an isocyanate, a cyanate, a thioisocyanate, a thiocyanate, a carbamate, an azide, a diazo group or a quinone group. Other groups which can be bonded to the cholesterol unit include an oligopeptide comprising from 2 to 20, preferably from 8 to 12, in particular 8, 10 or 12 amino acid residues, an oligonucleotide, or individual amino acids, monosaccharides, disaccharides or polysaccharides.

All the substances which have been formed from cholesterol by substituting or adding groups come within the term cholesterol derivatives. In particular, cholesterol derivatives are understood as being compounds which are formed from cholesterol in only one reaction step and are chemically closely related to cholesterol. Cholesterol derivatives include cholesterol sulfate, cholesterol thiosulfate and all cholesterol derivatives in which organic groups are substituted or added onto the cholesterol unit. Suitable substituted or added groups comprise: a halide atom, bonded to an alkyl, alkenyl, alkynyl or aryl radical, an alcohol group (primary, secondary or tertiary), an ether group, a carbonyl function (e.g. aldehyde or ketone), a carboxylic acid group, a carboxylic anhydride group, a carbamoyl group, a haloformyl group, a cyano group, an ester group, including a lactone group, a benzyl, phenyl, tolyl or tosyl, a sulfonyl group, an amino group (primary, secondary or tertiary), an isocyanate, a cyanate, a thioisocyanate, a thiocyanate, a carbamate, an azide, a diazo group or a quinone group. Other groups which can be bonded to the cholesterol unit include an oligopeptide comprising from 2 to 20, preferably from 8 to 12, in particular 8, 10 or 12, amino acid residues, an oligonucleotide, individual amino acids, monosaccharides, disaccharides or polysaccharides.

Examples of preferred cholesterol derivatives are cholesterol sulfate and cholesterol molecules which are derivatized on the OH function.

The present invention furthermore provides a process which can be used for monitoring and observing the direction of targeting and processing of the abovementioned active compounds, that is the gangliosides, gangloside derivatives and cholesterol derivatives, by attaching a detectable group, for

example a fluorescent group, to one of the previously enumerated compounds, for example by means of substitution or addition.

- 5 According to the invention, the term "modulation" covers any structural and chemical change in the microdomains, in particular a change in the structural constitution and/or in the combination of the individual constituents of the microdomains. For its  
10 part, the modulation of the sphingolipid-cholesterol microdomains preferably brings about a change in the membrane transport, signal transmission and/or cell adhesion properties and/or a change in enzymic processes. These effects are caused by using  
15 gangliosides and other substances for the modulation, that is, in particular, for altering the structure of sphingolipid-cholesterol microdomains. Thus, signal transmission properties can be influenced by using exogenously added gangliosides and other substances to  
20 alter the sphingolipid-cholesterol rafts. In addition to this, it has been observed that the addition of gangliosides to cells alters the degree of autophosphorylation of many kinases. The sphingolipid-cholesterol microdomains consequently constitute  
25 platforms for a variety of processes, in particular for signal transmission processes, which, according to the invention, can be influenced by using gangliosides to modulate the microdomains. Modulation of the sphingolipid-cholesterol microdomains can also prevent  
30 the phagocytosis of bacteria and parasites in mammalian cells. Modulation of the sphingolipid-cholesterol microdomains can also prevent the uptake of viruses in mammalian cells as well as their transport and release.
- 35 GPI-anchored proteins associate, for example, with tyrosine kinases of the Src family (Brown, Trends Cell Biol. 2 (1992), 338-342). In addition, cell signal proteins are concentrated in the caveolae, which are



plasma membrane invaginations which are involved in the sequestering and organizing of raft lipid domains. Many signal transmission molecules, such as PDGF (platelet derived growth factor), EGF (epidermal growth factor),  
5 protein kinase C and the insulin receptor, are associated with sphingolipid-cholesterol rafts or caveolae. The signal transmission pathways which are mediated by these molecules can be inhibited by the exogenous addition of gangliosides and other  
10 substances. It has now been observed that gangliosides modify these signal transmission pathways by, for example, effecting a cluster disassembly of protein-containing microdomains, in particular of GPI-AP-containing microdomains.

15 Still other signal transmission processes are dependent on the arrangement of lipid and protein components in the rafts. A modulation, in particular a disassembly of the rafts by the exogenous administration of  
20 gangliosides can be brought about, for example, in order to antagonize a microdomain-dependent signal transmission. These signal transmission processes include, inter alia, an antibody-induced patching of GPI-anchored PLAP (placental alkaline phosphatase),  
25 which leads to an accumulation of the Src-like tyrosine kinase fyn (Harder et al., J. Cell. Biol. 141 (1998), 929-942). Furthermore, physiological reactions which are induced by microdomain clusters have been described in the case of lymphocytes (Brown, Curr. Opin. Immunol.  
30 5 (1993), 349-354). Thus, an antibody-induced crosslinking of GPI-anchored proteins leads to the activation of T lymphocytes (Thomas et al., J. Biol. Chem. 267 (1992), 12317-12322), and the crosslinking of the IgE receptor FcεRI causes an allergic reaction  
35 including secretion of histamine in mast cells (Holowka et al., Ann. Rev. Biophys. Biomol. Struct. 25 (1996), 73-80; Field et al., J. Biol. Chem. 272 (1997), 4276-4280). Furthermore, gangliosides can be used for

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treating diseases which are connected to a cell movement, such as the control of tumors and/or metastases. Tumor cells use the abovementioned mechanism in order to evade the immune response. Raft-mediated processes, such as the activation of T cells in the presence of tumor cells, can consequently be influenced by gangliosides. The use, according to the invention, of gangliosides and other substances makes it possible to modulate, and thereby selectively influence, functions which are affected by sphingolipid-cholesterol microdomains.

It has furthermore been ascertained that an increase in the autophosphorylation of various protein kinases can be observed when gangliosides are added to cells.

In addition to this, the gangliosides and other substances can be used as agents for clarifying the pathogenesis of diseases which are associated with activities at sphingolipid-cholesterol microdomains. Thus, it has been recently demonstrated that a decrease in the cholesterol of hippocampus neurons inhibits the secretion of  $\beta$ -amyloid peptide, from which it follows that the sphingolipid-cholesterol microdomains play an important role in the proteolytic processing of the amyloid precursor protein (APP) in Alzheimer's disease (Simons et al., Proc. Natl. Acad. Sci. USA 95 (1998), 6460-6464). The transformation of the GPI-anchored prion protein into the scrapie protein isoform has likewise been suggested to be connected with sphingolipid-cholesterol microdomains (Taraboulos et al., J. Cell Biol. 129 (1995), 121-132). Preference is therefore given to using the gangliosides such that the modulation of the sphingolipid-cholesterol microdomains brings about a change in the proteolysis of the amyloid precursor protein in Alzheimer's disease or a modification of a prion protein.

The modulation, according to the invention, of sphingolipid-cholesterol microdomains can be of interest for both and therapeutic, preventive and diagnostic purposes. In this connection, the  
5 gangliosides are preferably administered in a dose of 3, preferably of at least 5 up to 30, and preferably up to 20 mg per kg per day.

In an in vitro system (cell culture), the dose which is  
10 used experimentally to elicit a reaction is 1 mg/ml for from about 10 to 12 hours. Taking into account the buffering effect of blood, plasma and cells in animals or humans, this indicates a dose range of from 3 to 30 mg/kg per day.

15 The invention is explained further by the following examples and the enclosed figures.

#### Figure 1

20 shows that gangliosides inhibit the crosslinking of GPI-anchored proteins, in particular of GH-DAF, in living cells. MDCK GH-DAF cells (MDCK: dog, cocker spaniel, kidney) were incubated, at 37°C for 1 hour, with 0, 10, 50 and 100 µM GM<sub>1</sub> (A) or with 10, 50 and  
25 100 µM bbG (B). CHO FR-GPI cells were incubated, at 37°C for 1 hour, with 100 µM bbG or with 100 µM GM<sub>1</sub>. The cells were subsequently crosslinked with BS<sup>3</sup>. The proteins were separated using 5-15% SDS-PAGE and, after Western blotting, detected using an anti-GH antibody,  
30 followed by ECL (electrochemoluminescence). The autoradiographs were scanned, and the intensity of the immunoreactive signal is depicted as relative optical density (ROD) for 0 µM GM<sub>1</sub> (A, upper curve), 0 µM bbG (B, upper curve), 100 µM GM<sub>1</sub> (A, lower curve) and 100 µM  
35 bbG (B, lower curve).

Figure 2

shows that gangliosides inhibit the crosslinking of FR-GPI in CHO cells. CHO FR-GPI cells were incubated, at 37°C for 1 hour, with 100 µM bbG or with 100 µM GM<sub>1</sub>.

- 5 The cells were subsequently crosslinked with BS<sup>3</sup>. The proteins were separated using 5-15% SDS-PAGE and detected, following Western blotting, using an anti-folate receptor antibody, followed by ECL (electrochemoluminescence).

10

Figure 3

shows the incorporation of GM<sub>1</sub> into the plasma membrane of MDCK GH-DAF cells. In (A), MDCK GH-DAF cells were incubated, at 37°C for 1 hour, with 1 µCi/ml tritium-containing GM<sub>1</sub> at a final ganglioside concentration of 100 µM and subsequently washed with BSA (bovine serum albumin) for 0, 10, 20, 30 or 45 minutes (hatched bars). The additive effect of a treatment with 0.1% trypsin for 5 minutes at 37°C, after the washing with BSA, is shown as solid bars. In (B), the crosslinking was carried out after incubating GM<sub>1</sub>-loaded cells with BSA for 0, 10, 20, 30 or 45 minutes. SDS-PAGE, Western blotting and detection were carried out as described in figure 1.

25

Figure 4

shows the increase in the detergent solubility of GH-DAF due to gangliosides. In (A), MDCK GH-DAF cells were loaded with 100 µM bbG for 1 hour at 37°C, or with 100 µM NBD-C<sub>6</sub>-HPC for 1 hour at 8°C, with 100 µM GM<sub>1</sub> for 1 hour at 37°C or with 100 µM bbG for 1 hour followed by a 6-hour incubation with DMEM-containing serum (A) or a treatment with 10 mM methyl-β-cyclodextrin for 1 hour at 37°C or an incubation with anti-GH for 1 hour at 12°C, followed by an incubation with a secondary antibody (B). The cells were extracted with Triton X-114 at 4°C for 30 minutes, and centrifuged, and the

35

shows that treatment with a ganglioside does not alter the immunofluorescence pattern of GH-DAF. MDCK GH-DAF cells were incubated, at 37°C for 1 hour, without any addition (A, D), with 100 μM bbG (B) or with 10 mM CD (C). The cells were fixed and labeled with an anti-GH antibody, followed by a Cy3 conjugated anti-sheep IgG (A, B and C), or crosslinked, prior to being fixed, in an antibody-induced manner.

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Figure 8

shows that the loading of MDCK GH-DAF cells with gangliosides alters the autophosphorylation status. MDCK GH-DAF cells were loaded with bbG or GM<sub>1</sub> at 37°C for 1 hour or crosslinked with 0.5 mM BS<sup>3</sup> at 4°C for 45 minutes. After lyzing, aliquots of the samples were separated on a 5-15% gel and the in-gel assay was carried out for the protein kinase activity. Bands whose intensity increased in association with the ganglioside treatment are marked with an arrow. Bands which only became visible after treatment with a ganglioside are marked with an arrow head.

Figure 9

shows the inhibition, by gangliosides, of the copatching of influenza HA and GH-DAF. MDCK GH-DAF cells were infected with the influenza HA virus and then incubated, at 37°C for 1 hour, with DMEM (A-F) or 100 µM GM<sub>1</sub> in DMEM (G-L). Following a subsequent treatment with a mixture of monoclonal anti-HA mouse antibodies and polyclonal anti-GH sheep antibodies, at 4°C, the patching was detected using labeled secondary Cy-3-anti-sheep (red) and FITC-anti-mouse (green) antibodies. The panels in the left-hand column show the distribution of GH while those in the middle column show the distribution of HA and those in the right-hand column show the combination of both signals. Panels D-F in each case show a detail from panels A-C while panels J-L show a detail from panels G-I. The lines in F and L correspond to 2 µM, while those in C and I correspond to 8 µM.

In order to investigate the influence of gangliosides on sphingolipid-cholesterol rafts, MDCK GH-DAF cells were loaded, at 37°C for 1 hour and in a serum-free medium, with various concentrations of GM<sub>1</sub> and crosslinked with BS<sup>3</sup>. As can be seen in figure 1A, treating MDCK GH-DAF cells with GM<sub>1</sub> decreased the quantity of crosslinked GH-DAF significantly. Quantifying the immunoreactive bands showed that 73% ± 6% of the GH-DAF formed oligomers in untreated cells, while 65% ± 7%, 57% ± 7% and 49% ± 7% of GH-DAF was found to be in crosslinked species in cells which had been treated with 10, 50 and 100 μM GM<sub>1</sub>, respectively. Incubating the cells with bovine brain gangliosides (bbG) also efficiently inhibited the formation of GH-DAF oligomers (figure 1B). When 100 μM bbG was used, the crosslinking efficiency was 51% ± 5%, as compared with 73% ± 6% in untreated cells.

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In order to determine whether the ability of gangliosides to decompose GPI-AP-containing rafts is independent of the cell type, CHO cells which were permanently expressing the GPI-anchored folate receptor (FR-GPI) were treated in the same way. The CHO FR-GPI cells were cultured in a folate-free Hams F-12 medium which contained 5% FCS, hygromycin (100 µg/ml), penicillin (100 U/ml) and streptomycin (100 µg/ml). The crosslinking of CHO FR-GPI cells with BS<sup>3</sup> leads to the appearance of crosslinked FR-GPI products, with a crosslinking efficiency of 28% ± 3% being observed. When CHO FR-GPI cells were loaded with 100 µM GM<sub>1</sub> or 100 µM bbG prior to the crosslinking, the crosslinking efficiency was decreased to 2.7% ± 2.5% and 3.3% ± 2%, respectively (see figure 2).

In order to quantify the quantity of GM<sub>1</sub> which is incorporated into the plasma membrane, MDCK GH-DAF cells were incubated, for 1 hour, with 1 µCi/ml tritium-containing GM<sub>1</sub> at a final ganglioside concentration of 100 µM. For this, GM<sub>1</sub> was extracted, purified in accordance with known methods (Tettamanti et al., Biochim. Biophys. Acta 296 (1973), 160-170), and radiolabeled at the C-3 of the long-chain base group (Sonnino et al., J. Lipid Res. 25 (1984), 620-629). The homogeneity of the radioactive compound <sup>3</sup>H-GM<sub>1</sub> was greater than 99% and its specific radioactivity was 1.31 Ci/mmol. The cells were incubated for 1 hour in DMEM which contained 1 µCi/ml tritium-containing GM<sub>1</sub> at a final ganglioside concentration of 100 µM, after which the cells were washed with PBS/BSA and subsequently incubated at 37°C, for from 0 to 45 minutes, with PBS/BSA. The cells were then treated for 5 minutes, at 37°C, with 1 ml of PBS which contained 0.1% trypsin. The cell lysates were analyzed for their content of radioactive ganglioside using a liquid scintillation counter (Beckmann



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Instruments, CA). The protein was determined using a protein assay reagent (BCA; Pierce, UK).

Only about 0.7% of the exogenously added ganglioside associated with the cells. It was possible to remove approximately 35% of the total associated GM<sub>1</sub> by incubating the cells with BSA solution (figure 3A). By subsequently treating the cells with trypsin, it was possible to detect two pools of associated GM<sub>1</sub>. About 30% of the remaining GM<sub>1</sub> was released by treating with trypsin (trypsin-labile pool) while about 70% was stable (trypsin-stable pool) (figure 3A). It is assumed that the trypsin-stable pool of GM<sub>1</sub> corresponds to the molecules which are inserted into the plasma membrane, whereas the trypsin-labile pool consists of gangliosides which interact with proteins which protrude from the plasma membrane (cf. Masserini et al., Biochemistry 29 (1990), 697-701; Saqr et al., J. Neurochem. 61 (1993), 495-411). The trypsin-stable pool of GM<sub>1</sub> contained, in an enriched state, that fraction which was resistant to an extraction with Triton X-100 at 4°C. After incubating cells with 100 µM tritium-containing GM<sub>1</sub>, 3.74 nmol of GM<sub>1</sub> (per mg of protein) were found in the detergent-insoluble fraction while 1.26 nmol of GM<sub>1</sub> were isolated from the soluble fraction, an observation which demonstrates that exogenous GM<sub>1</sub> accumulates in the rafts.

In order to exclude the possibility that the inhibition of the crosslinking of GH-DAF takes place as the result of the effect of gangliosides which are loosely associated with the surface, the crosslinking was carried out on GM<sub>1</sub>-loaded cells before and after incubating with BSA. Under these two conditions, the crosslinking efficiency remained the same (see figure 3B; the variation in efficiency was less than 5% under all conditions). Consequently, gangliosides which are firmly associated with the membrane are responsible

for the inhibition of the crosslinking. In accordance with the criterion used by other authors (Kanda et al., J. Biochem. (Tokyo) 91 (1982), 1707-1718; Schwarzmann et al., Biochemistry 22 (1983), 5041-5048) it was established that 70% of the firmly associated GM<sub>1</sub> was incorporated into the double layer. Consequently, the contribution made by the trypsin-labile pool to the crosslinking, if it occurs at all, is very small in particular since the solubility of GH-DAF in TX-114 was substantially increased by gangliosides. This behavior cannot be explained by ganglioside micelles which bind to protruding proteins.

#### Example 1A

Using cholesterol sulfate to disrupt cholesterol-enriched microdomains in MDCK cells

Cholesterol sulfate-methyl- $\beta$ -cyclodextrin (M $\beta$ CD) inclusion complexes were prepared essentially as described by Klein et al. (1995), Biochemistry 34, p. 13784. 5 ml of an aqueous solution of M $\beta$ CD (10 mg/ml) were stirred constantly, at 60°C, on a water bath and 0.15 ml of a cholesterol sulfate (CS) solution (10 mg/ml in isopropanol) was added in small portions. The solution was ultrasonicated at 40°C for 10 minutes, centrifuged at 100 000 g for 10 minutes and lyophilized. The dried inclusion complex was finally dissolved in 300  $\mu$ l of water in order to yield a stock solution which was 10 mM with respect to cholesterol sulfate. MDCK GH-DAF cells (10<sup>6</sup>) were inoculated into a 35 mm culture dish and the cells were grown to high density overnight. After having been washed twice with PBS, the cells were incubated at 37°C for 1 hour in DMEM together with cholesterol sulfate-M $\beta$ CD inclusion complexes. The cells were washed three times with ice-cold PBS (phosphate-buffered saline solution)/BSA (bovine serum albumin) (PBS containing 2 mg of BSA/ml) in order to remove inclusion complexes and the cells

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were cooled on ice for 5 minutes before they were subjected to crosslinking. Routinely, in a standard crosslinking protocol, the membrane-impermeable crosslinker bis(sulfosuccinimidyl)suberate (BS<sup>3</sup>) is used, diluted down to 0.5 to 1 mM, in PBS. Cells were incubated with a BS<sup>3</sup> solution at 4°C for 45 minutes. Unreacted crosslinking agent was quenched, at 4°C for 15 min, with 1 mM glycine dissolved in PBS. The cells were subsequently lysed with a Triton X-114-containing buffer and a detergent-rich phase was prepared in order to enrich GPI anchor proteins. The proteins were precipitated from the detergent-rich phase, fractionated on an SDS-PAGE gradient gel and blotted onto nitrocellulose, and GH-DAF was visualized using anti-GH antibodies, HRP-conjugated secondary antibodies and ECL.

The crosslinking of GH-DAF was significantly decreased, in a similar manner to that occurring in association with GM<sub>1</sub> treatment.

### Example 2

#### Using gangliosides to alter the detergent solubility of GH-DAF

A frequently employed criterion for investigating the association of a protein with microdomains is its resistance to extraction by nonionic detergents, such as Triton X-100, at 4°C (Brown et al., Cell 68 (1992), 533-544). The detergent solubility of GH-DAF was therefore investigated after the cells had been loaded with gangliosides. It was found that the solubility of GH-DAF in TX-114 was significantly increased from 51 ± 4% to 83% ± 10% and 76% ± 8% in GM<sub>1</sub>-loaded cells and in bbG-loaded cells, respectively (figure 4A).

These results demonstrated that gangliosides which are inserted into the plasma membrane displace GPI-APs from

lipid microdomains. Consequently, gangliosides which are bound to proteins which are protruding from the surface of the plasma membrane either do not exert an influence on, in particular inhibit, the crosslinking by means of steric hindrance, or only do this to a very slight extent. It is known that a decrease in the cholesterol content in cell membranes increases the solubility of GPI-APs in nonionic detergents (Cerneus et al., J. Biol. Chem. 268 (1993), 3150-3155; Hanada et al., J. Biol. Chem. 270 (1995), 6254-6260; Scheiffele et al., EMBO J. 16 (1997), 5501-5508). The solubility of GH-DAF in association with an extraction with TX-114 was shifted to  $80\% \pm 7\%$  after the membrane cholesterol had been extracted with CD (figure 4B). By contrast, stabilizing the microdomains by means of an antibody-induced crosslinking reduces the solubility of GH-DAF down to  $34\% \pm 7\%$  (figure 4B).

The results demonstrate that using exogenous gangliosides to modulate or alter the lipid content in the cell membrane modulates both the cluster behavior of GPI-APs and their ability to be solubilized in TX-114 at  $4^{\circ}\text{C}$ .

### 25 Example 3

#### Effect of gangliosides on microdomains

As was demonstrated above, loading MDCK-GH-DAF cells with gangliosides displaces GPI-APs from the rafts. In order to exclude the possibility that this effect arises partially as a result of detergent-like properties possessed by gangliosides, use was made of n-octyl- $\beta$ -D-glucopyranoside (OG). This detergent resembles gangliosides chemically (it contains a carbohydrate head group, for example) and is known to solubilize GPI-APs completely when it is used above the critical micelle concentration (20-25 mM). MDCK GH-DAF cells were incubated with varying concentrations of OG

and crosslinked with BS<sup>3</sup>. No influence on the crosslinking of GH-DAF was observed when OG was used at the same concentrations as the gangliosides (10-100  $\mu$ M). Only a minimal inhibition of the crosslinking was observed even when cells were incubated with an 100-fold higher concentration of OG (10 mM) (figure 5; the inhibition amounted to 3.5%  $\pm$  0.7%). Remarkably, the membranes remained intact even under these circumstances since caveolin-1, which is a protein membrane whose N terminus and C terminus are both arranged towards the cytoplasm, was not crosslinked. It was only when OG was used above its critical micelle concentration (20 mM) that caveolin-1 was found as a crosslinked product of high molecular weight. The treatment of the MDCK GH-DAF cells with 100  $\mu$ M GM<sub>1</sub> did not lead to caveolin-1 becoming crosslinked. These data show that the inhibition, by gangliosides, of the crosslinking of GH-DAF does not occur due to the membrane being solubilized as a result of any detergent-like properties possessed by gangliosides.

#### Example 4

#### Using gangliosides to inhibit the crosslinking of GH-DAF

This example demonstrates that inhibiting the clustering of GPI-anchored proteins is a specific property of gangliosides. A lipid which is not a constituent of the sphingolipid-cholesterol microdomains should not have any effect on the crosslinking and solubility of GH-DAF. Ideally, phosphatidyl choline molecules, such as those which are found in cell membranes, for example, should be used for such a control experiment. However, these lipids cannot be introduced into the membrane by adding them exogenously. For this purpose, therefore, MDCK GH-DAF cells were incubated with a partially water-soluble,

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fluorescent-labeled analog of phosphatidyl choline (NBD-C<sub>6</sub>-HPC). NBD-C<sub>6</sub> lipids are known to reach the plasma membrane rapidly at low temperature, and their accumulation in the plasma membrane can be easily  
5 observed by means of immunofluorescence microscopy (Kean et al., J. Cell. Biol. 123 (1993), 1403-1419). The insolubility of NBD-C<sub>6</sub> HPC in detergents was then investigated. Cells were loaded with NBD-C<sub>6</sub>-HPC and a  
10 detergent extraction was carried out at 4°C using Triton X-114. More than 99% of the NBD-C<sub>6</sub>-HPC was isolated from the detergent-soluble fraction, thereby indicating that the lipid is not associated with the microdomains. No change in the crosslinking pattern was observed after incubating with various concentrations  
15 of NBD-C<sub>6</sub>-HPC. The crosslinking efficiency was 73% ± 5% in cells treated with 100 µM NBD-C<sub>6</sub>-HPC as compared with 73% ± 6% in control cells (figure 6A). NBD-C<sub>6</sub>-HPC did not alter the solubility of GH-DAF (54% ± 2% soluble), either (figure 4A).

20 In order to determine whether the inhibition of the crosslinking of GH-DAF with gangliosides is reversible, MDCK GH-DAF cells were loaded with 100 µM bbG and subsequently incubated for 6 hours in a serum-  
25 containing medium prior to the crosslinking. As shown in figure 6B, 6 hours of incubation with a serum-containing medium were sufficient in order to restore the crosslinking pattern of GH-DAF almost completely. The crosslinking efficiency was 6% ± 1% less than in  
30 the control cells.

The insolubility of GH-DAF in the detergent TX-114 was likewise restored by incubating bbG-loaded cells with a serum-containing medium for 6 hours (61% ± 4% soluble)  
35 (figure 4A).

This example shows that the inhibition of the crosslinking of GH-DAF by gangliosides is specific and reversible.

5 Example 5

The distribution of GH-DAF is not altered by gangliosides

Sphingolipid-cholesterol microdomains or rafts are  
10 small and highly dynamic structures which cannot be resolved by conventional microscopic techniques. An examination was carried out to determine whether loading the cells with gangliosides leads to a global rearrangement of the plasma membrane and thus of the  
15 GPI-APs. The distribution of GH-DAF on MDCK cells, with or without loading with bbG, was analyzed by means of immunofluorescence labeling. In order to prevent a redistribution of GH-DAF after the fixing had taken place, a formaldehyde fixing was used, with this fixing  
20 being combined with a subsequent fixing with methanol (Harder et al., J. Cell. Biol. 141 (1998), 929-942). Incubating the cells with 100  $\mu$ M bbG for 1 hour prior to the fixing, and labeling with antibody, had no effect on the diffuse distribution of GH-DAF on the  
25 cell surface (figures 7A and B). The intracellular staining stems from the arrangement of the protein in the Golgi apparatus and/or from the endosomal arrangement. A decrease in the membrane content of cholesterol, effected using 10 mM CD, did not lead to  
30 any detectable changes in the distribution of GH-DAF, either (figure 7C). By comparison, the addition of anti-GH-DAF antibodies before the fixing took place led to a speckled distribution of the immunofluorescence signal (figure 7D). These results demonstrate that the  
35 inhibition of the crosslinking by gangliosides does not occur because of any rearrangement of the membrane.

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Example 6

Loading cells with gangliosides alters the autophosphorylation state of protein kinases

5 This example demonstrates that there is a correlation between the administration of gangliosides to MDCK GH-DAF cells and cell signal transmission processes. As an indication of signal transmission, the autophosphorylation state of cell protein kinases was  
10 measured, using an in-gel assay, after cells had been loaded with gangliosides. The conditions for the ganglioside loading were the same as those for the investigations on crosslinking. As can be seen from figure 8, the intensity of various bands increased  
15 significantly after adding gangliosides (arrow) whereas other bands only became visible following treatment with a ganglioside (arrow heads). In this connection, it is to be noted that chemical crosslinking with BS<sup>3</sup> on its own does not lead to any significant changes in  
20 the autophosphorylation state. These experiments demonstrate that, in addition to modulating the association of GPI-anchored proteins with microdomains, gangliosides are able to induce signal transmission processes in MDCK cells.

25

Example 7

Using gangliosides to inhibit the copatching of influenza HA and GH-DAF

30 Sphingolipid-cholesterol microdomains are small and highly dynamic structures which cannot be resolved by conventional microscopic techniques. An investigation was carried out to determine whether loading the cells with gangliosides leads to a redistribution of GPI-APs.  
35 The distribution of GH-DAF on MDCK cells, with or without loading with bbG, was analyzed by means of immunofluorescence labeling. In order to prevent a redistribution of GH-DAF after the fixing had taken



place, a formaldehyde fixing was used, with this fixing being combined with a subsequent fixing with methanol (Harder et al., J. Cell. Biol. 141 (1998), 929-942). Incubating the cells with 100  $\mu$ M bbG for 1 hour prior to the fixing, and labeling with antibody, had no effect on the diffuse distribution of GH-DAF on the cell surface (figure 8, panels A and B). The intracellular staining stems from the arrangement of the protein in the Golgi apparatus and/or from the endosomal arrangement. A decrease in the membrane content of cholesterol, effected using 10 mM CD, did not lead to any detectable changes in the distribution of GH-DAF, either (figure 7C). By comparison, the addition of anti-GH-DAF antibodies before the fixing took place led to a patch-like distribution of the immunofluorescence signal (figure 7D).

Incubating living cells simultaneously with antibodies directed against microdomain-associated proteins leads to the independently crosslinked components being redistributed into overlapping patches. However, copatching is only observed in the case of raft marker pairs and not in the case of pairs consisting of a raft marker and a non-raft marker; it can be concluded from this that attractive forces are present in the shared lipid environment of raft proteins.

An investigation was now carried out to determine whether the exogenous administration of gangliosides disturbs the physical linkage of GH-DAF to lipid microdomains. The trimeric transmembrane protein influenza hemagglutinin (HA) was expressed, as a protein marker associated with the microdomains, in MDCK GH-DAF cells.

The cells were incubated simultaneously, at 4°C, with a monoclonal anti-HA mAb and a polyclonal anti-GH Ab, followed by an incubation with the respective secondary

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antibodies. A microscope analysis showed that HA and GH-DAF were copatching in the majority of the cells (60% copatching, 35% partial overlapping and 5% random distribution) (figures 9A-F). When the cells were incubated for 1 hour with 100  $\mu$ M GM<sub>1</sub> prior to the antibody-induced crosslinking, only a partial coclustering of HA and GH-DAF was then observed in most of the cells (31% coclustering, 50% partial overlapping and 19% random distribution) (figures 9G-L). These results demonstrate that the attractive forces between patches of HA and GH-DAF are disturbed by administering gangliosides, showing that GPI-APs are displaced out of the lipid microdomains.

#### 15 Example 8

##### Experimental procedures

The following procedures were used in the above examples:

20

**Inserting lipids, reducing the content of cholesterol and treating cells with octyl glucoside:**

Stock solutions of bovine brain gangliosides (bbG) or GM<sub>1</sub> (1-10 mM) were prepared in PBS. The cells were loaded with 10-100  $\mu$ M gangliosides in DMEM at 37°C for 1 hour (Masserini et al., Biochemistry 29 (1990), 697-701; Saqr et al., J. Neurochem, 61 (1993), 395-411). In order to remove excess lipid, cells were washed thoroughly with PBS which contained 2 mg of defatted BSA/ml (PBS/BSA). An ethanolic 0.5% stock solution of 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (NBD-C<sub>6</sub>-HPC) was injected into PBS, while vortexing vigorously, in order to give a 1 mM solution. The insertion of NBD-C<sub>6</sub>-HPC into membranes was carried out, at 8°C for 1 hour, using 10-100  $\mu$ M NBD-C<sub>6</sub>-HPC. In order to free MDCK GH-DAF cells from the cholesterol,

The recently described crosslinking protocol was used (Friedrichson et al., Nature 394 (1998), 802-805). In brief, cells were washed twice with cold PBS. The crosslinking was carried out at 4°C for 45 minutes using 0.5 mM BS<sup>3</sup>. Unreacted crosslinker was quenched, at 4°C for 15 minutes, with 50 mM glycine. The cells were lysed, at 4°C for 20 minutes and at 37°C for 10 minutes, in a TX-114 lysis buffer (150 mM NaCl, 10 mM tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton-X-114 and protease inhibitors). The lysates were cooled briefly on ice and clarified by being centrifuged for 15 minutes at 15 000 g. The supernatants were subjected, at 37°C for 5 minutes, to a temperature-induced phase separation. Aqueous and detergent-enriched phases were separated by centrifuging at 13 000 rpm for 3 minutes and at room temperature (RT). The aqueous phases were discarded and 0.9 ml of TX-114 washing buffer (150 mM

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NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.06% Triton-X-114 and protease inhibitors) was added to the detergent phases. After a 15-minute centrifugation at 15 000 g and 4°C, the samples were subjected to a further phase separation. Finally, the detergent phases were precipitated with cold acetone (-20°C) and boiled in a Lämmli sample buffer at 95°C for 5 minutes. The proteins were separated using a 5-15% SDS-PAGE and transferred to nitrocellulose. Polyclonal antibodies directed against GH and the folate receptor, followed by the corresponding secondary antibodies and ECL, were used in order to detect GH-DAF and FR-GPI. Immunoreactive bands were quantified by scanning films densitometrically or using a LAS-1000 Luminescence Image Analyzer (Fujifilm, Germany). The data for each condition were averaged and the fluctuation expressed as a standard deviation. The experiments were carried out from 3 to 6 times. In the case of some gels, the corresponding X-ray films were scanned using Fotoshop software and the optical density of the immunoreactive bands was determined and printed out using MacBas (version 2.0).

**Immunofluorescence:**

Cells cultured on cover slips were washed 3 times with PBS and fixed with 3.7% paraformaldehyde in PBS at 8°C for 6 minutes and then with methanol at -20°C for 10 minutes. Subsequently, the cells were incubated with an anti-GH antibody in PBS at room temperature for 30 minutes, followed by an incubation with Cy3-conjugated anti-sheep-IgG at room temperature for 30 minutes. Three washing steps with PBS were carried out after each of the above incubations. The cover slips were embedded in Moviol and photographs were taken using a high-resolution C 4742-95 digital camera (Hamamatsu Photonics K.K., Japan), and the digital deconvolution was carried out using the digital

confocal Openlab routine (version 1.7.7) (Improvision, UK).

For the antibody-induced crosslinking experiments, the  
5 anti-GH antibody and the Cy3-conjugated anti-sheep-IgG  
were diluted in DMEM. The cells were incubated with the  
anti-GH antibody at 37°C for 20 minutes, washed with  
DMEM and incubated with Cy3-conjugated anti-sheep-IgG  
at 37°C for 20 minutes. The cells were fixed and  
10 embedded in Moviol as described above.

#### **In-gel protein kinase assay:**

Cells cultured in 6-well dishes were loaded with  
gangliosides or crosslinked, as described above, with  
15 BS<sup>3</sup>, cooled on ice, washed 3 times with ice-cold PBS  
and lysed, at 4°C for 30 minutes, in 400 µl of lysis  
buffer (20 mM tris-acetate, pH 7.0, 0.1 mM EDTA, 1 mM  
EGTA, 1 mM sodium orthovanadate, 10 mM β-glycero-  
phosphate, 50 mM sodium fluoride, 5 mM sodium  
20 pyrophosphate, 1% TX-100, 1 mM benzamidine, 2 µg of  
leupeptin/ml, 0.1% β-mercaptoethanol, 0.27 M sucrose  
and 0.2 mM PMSF) and scraped off using a rubber  
policeman. The lysates were clarified at 15 000 g for  
15 minutes and aliquots were boiled, at 95°C for  
25 5 minutes, with 5 × sample buffer. The samples were  
fractionated on 5-15% gels which had been polymerized  
with 5 mg of BSA/ml. The in-gel protein kinase assay  
was carried out in accordance with the method described  
by van Dam (van Dam et al., EMBO J. 14 (1995),  
30 1798-1811) apart from the fact that 65 µCi of γ-<sup>32</sup>P-ATP  
in 3 ml of buffer K were used per gel.

#### **Viral infection and antibody-induced patching:**

For an infection with the HA influenza virus, the virus  
35 was diluted in an infection medium (MEM, 50 mM Hepes,  
pH 7.3, penicillin (100 U/ml)/streptomycin (100 µg/ml),  
0.2% BSA), and virus adsorption was carried out for  
1 hour. The infection was continued for a further

2.5 hours. 100  $\mu$ M GM<sub>1</sub>, diluted in DMEM, were added to some cells during the last hour. For an antibody-induced patching of HA and GH-DAF, the cells were incubated with a polyclonal anti-GH antibody and a monoclonal anti-HA PR8 (H17L10), diluted 1:3 000 and 1:50, respectively, in DMEM. The incubation was carried out at 4°C for 1 hour. After a brief washing with PBS/0.2% BSA, the cells were incubated, at 4°C for 1 hour, with the corresponding FITC-coupled and Cy-3 coupled secondary antibodies. The cells were fixed and embedded and photographs were taken as described above. For the quantification, 60 randomly selected cells from two different experiments were stored as digital images. The cells were subdivided into three different categories in accordance with the extent of the overlapping between the patches: (1) coclustering (more than 70% overlapping); (2) partial coclustering; (3) random distribution.

The invention consequently relates in particular to the use of gangliosides and other substances for modulating sphingolipid-cholesterol microdomains. It can be used, for example, for developing active compounds which exert an influence on membrane transport, signal transmission, cell adhesion and/or enzymic processes in mammalian cells. The modulation can also prevent the entry of bacteria, pathogens and viruses into the cells.

## Patent claims

1. The use of gangliosides, ganglioside derivatives  
and/or cholesterol derivatives for preparing an  
5 agent for modulating sphingolipid-cholesterol  
microdomains.
2. The use as claimed in claim 1,  
characterized in that  
10 the agent influences the location of components  
and their function on the sphingolipid-cholesterol  
microdomains.
3. The use as claimed in claim 1 or 2,  
15 characterized in that  
the agent influences the location of proteins on  
the sphingolipid-cholesterol microdomains.
4. The use as claimed in claim 3,  
20 characterized in that  
the agent influences the location of anchor  
proteins, acylated proteins, Src kinases and/or  
cholesterol-anchored proteins and other raft  
proteins.
- 25 5. The use as claimed in claim 3 or 4,  
characterized in that  
the agent acts on glycosylphosphatidylinositol  
anchor proteins, kinases of the Src family,  
30 influenza virus hemagglutinin and other viral  
proteins and/or caveolin-1, 2 or 3 in the  
sphingolipid-cholesterol microdomain.
6. The use as claimed in one of the preceding claims,  
35 characterized in that  
the agent brings about a disassembly of the  
protein clusters.

7. The use as claimed in one of the preceding claims, characterized in that the ganglioside is selected from bovine brain gangliosides, GM<sub>1</sub>, GD1a, GD1b, GD3, GM2, GM3, GQ1a, GQ1b and/or globosides and their derivatives, in particular unsaturated sphingosines or ceramides containing unsaturated or short fatty acids.
- 10 8. The use as claimed in one of the preceding claims, characterized in that cholesterol derivatives, in particular cholesterol sulfate, are employed.
- 15 9. The use as claimed in one of the preceding claims, characterized in that the modulation of the sphingolipid cholesterol microdomains brings about a change in membrane transport, signal transmission and/or cell adhesion properties and/or in enzymic processes.
- 20 10. The use as claimed in one of the preceding claims, characterized in that the modulation of the sphingolipid-cholesterol microdomains brings about a change in the proteolysis of the amyloid precursor protein of Alzheimer's disease or a modification in a prion protein.
- 25 11. The use as claimed in one of the preceding claims, characterized in that the modulation of the phingolipid-cholesterol microdomains prevents the phagocytosis of bacteria and parasites in mammalian cells.
- 30 12. The use as claimed in one of the preceding claims, characterized in that
- 35



the modulation of the sphingolipid-cholesterol microdomains prevents the uptake of viruses into mammalian cells and/or their transport and release.

5

13. The use as claimed in one of the preceding claims, characterized in that the agent is employed as a therapeutic agent.

- 10 14. The use of gangliosides, ganglioside derivatives and/or cholesterol derivatives for modulating sphingolipid-cholesterol microdomains.

- 15 15. A process for modulating sphingolipid-cholesterol microdomains, characterized in that gangliosides, ganglioside derivatives and/or cholesterol derivatives are administered to a patient at a dose of from 3 mg to 30 mg per kg per day.

**Abstract**

The use of gangliosides and other substances for preparing an agent for modulating sphingolipid-cholesterol microdomains is described.

Wr/ANM/20597PWO September 28, 2000

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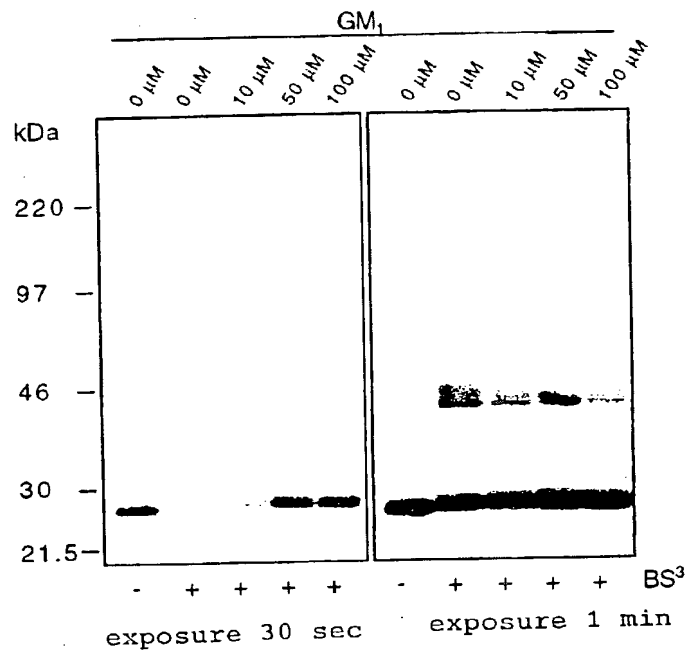
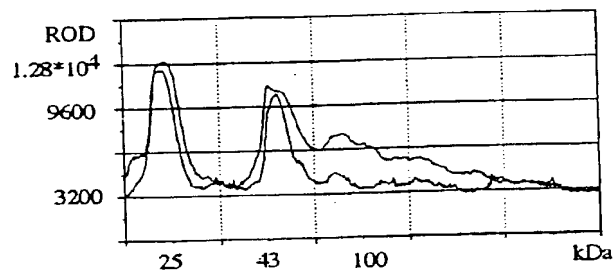


Fig. 1A



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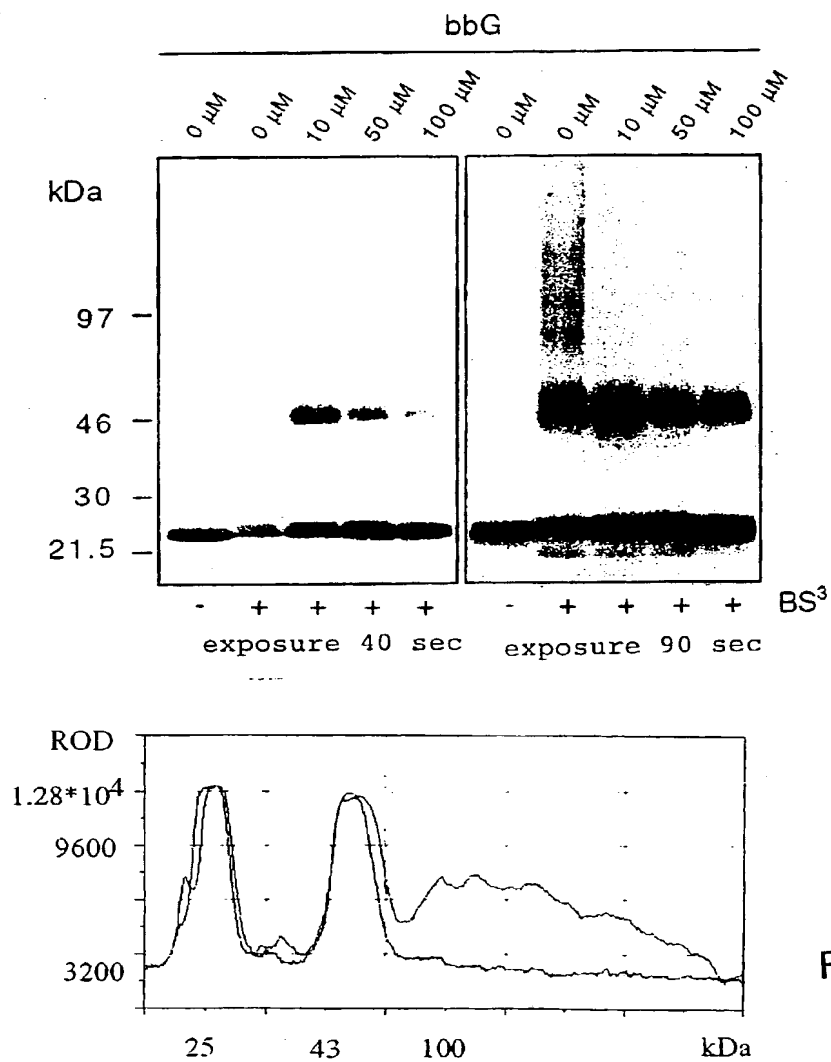


Fig. 1B

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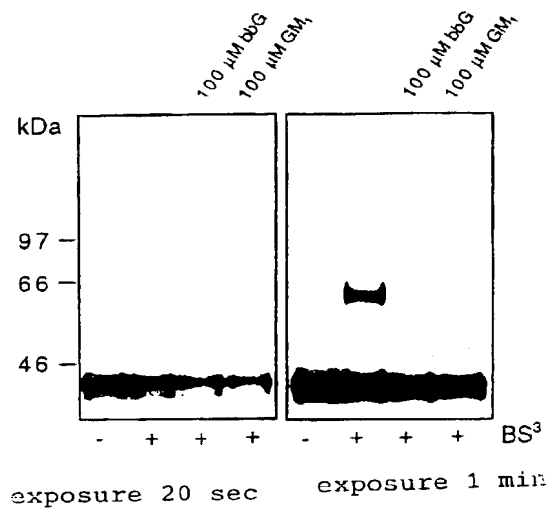


Fig. 2

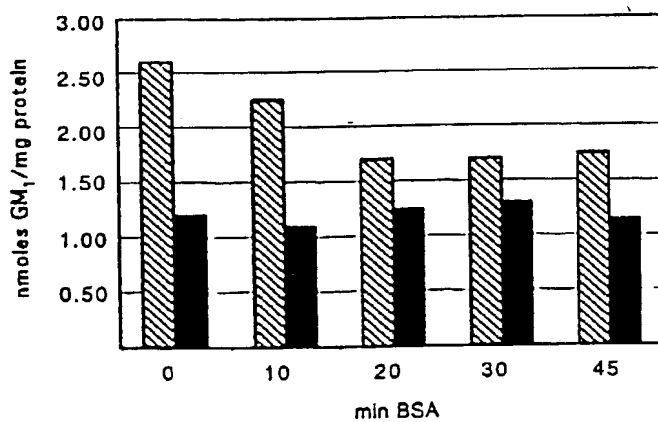


Fig. 3A

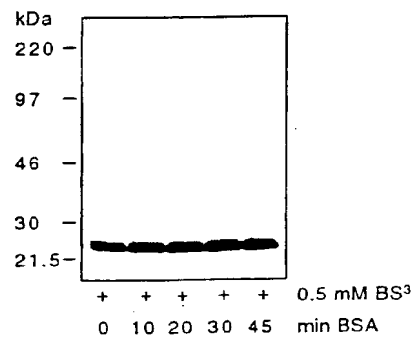


Fig. 3B

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A

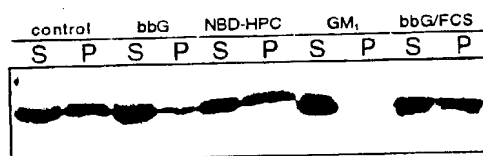


Fig. 4A

B

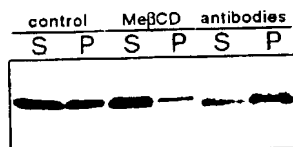


Fig. 4B

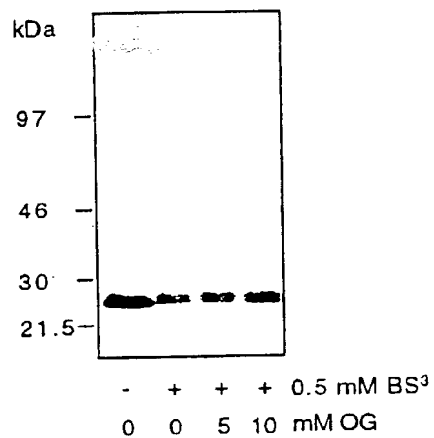


Fig. 5

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Fig. 6A

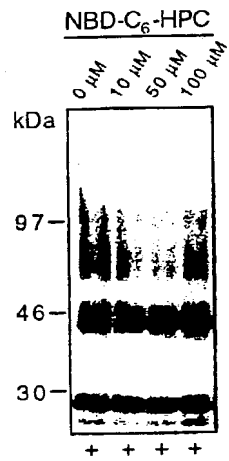


Fig. 6B

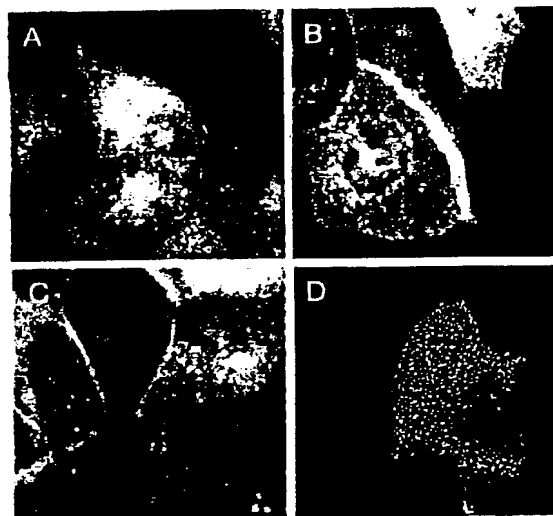
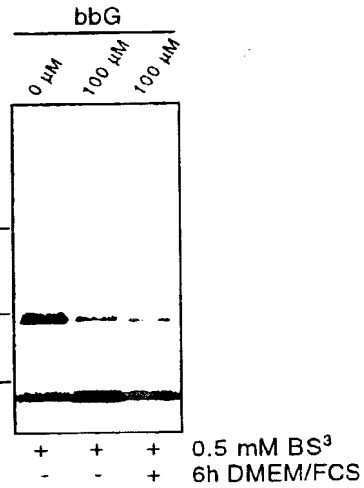


Fig. 7

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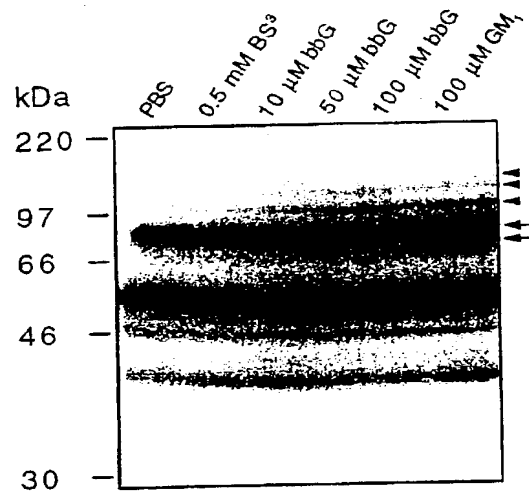


Fig. 8



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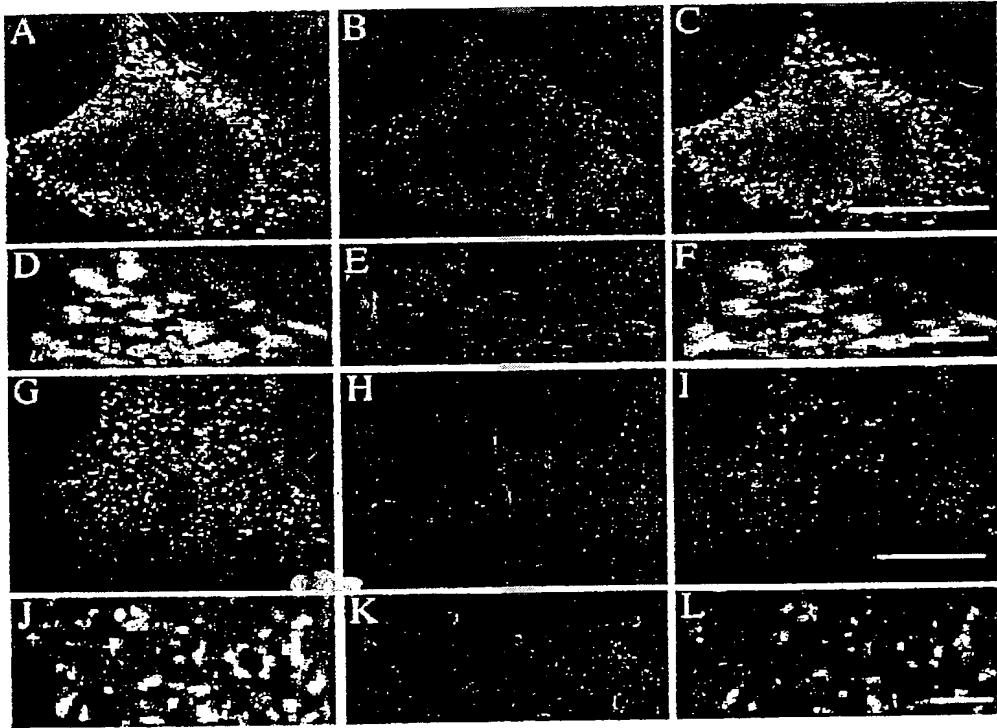


Fig. 9

## Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

(Insert Title) Use of gangliosides and other substances for modulating sphingolipid-cholesterol microdomains

the specification of which is attached hereto unless the following box is checked:

☒ was filed on September 29, 2000 as PCT International Application  
Number PCT/EP00/09588 and was amended on \_\_\_\_\_  
and/or was filed on \_\_\_\_\_ as United States Application  
Number \_\_\_\_\_ and was amended on \_\_\_\_\_

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International Application having a filing date before that of the application(s) for which priority is claimed:

| (List prior foreign applications. See note A on back of this page) | <u>199 46 737.4</u><br>(Number) | <u>DE</u><br>(Country) | <u>Sept 29, 1999</u><br>(Day/Month/Year Filed) | Priority Claimed<br><input checked="" type="checkbox"/> Yes <input type="checkbox"/> No |
|--|---------------------------------|------------------------|--|---|
|  | _____<br>(Number)               | _____<br>(Country)     | _____<br>(Day/Month/Year Filed)                | <input type="checkbox"/> Yes <input type="checkbox"/> No                                |
|  | _____<br>(Number)               | _____<br>(Country)     | _____<br>(Day/Month/Year Filed)                | <input type="checkbox"/> Yes <input type="checkbox"/> No                                |

I hereby claim the benefit under 35 U.S.C. §119(c) of any United States provisional application(s) listed below.

|                               |                        |
|-------------------------------|------------------------|
| _____<br>(Application Number) | _____<br>(Filing Date) |
| _____<br>(Application Number) | _____<br>(Filing Date) |

(See Note B on back of this page)

☐ See attached list for additional prior foreign or provisional applications.

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) (U.S. or PCT) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(List prior U.S. Applications or PCT International applications designating the U.S.)

|                                   |                        |  |
|-----------------------------------|------------------------|--|
| _____<br>(Application Serial No.) | _____<br>(Filing Date) | _____<br>(Status) (patented, pending, abandoned) |
| _____<br>(Application Serial No.) | _____<br>(Filing Date) | _____<br>(Status) (patented, pending, abandoned) |

And I hereby appoint as principal attorneys: Robert B. Murray, Reg. No. 22,980; Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,934; Douglas H. Goldhush, Reg. No. 33,125; David T. Nikaido, Reg. No. 22,663; Monica Chin Kius, Reg. No. 36,105; Richard J. Berman, Reg. No. 39,107; King L. Wong, Reg. No. 37,500; James A. Poulos, III, Reg. No. 31,714; Patrick D. Muir, Reg. No. 37,403; Murat Ozgu, Reg. No. 44,275; Bradley D. Goldizen, Reg. No. 43,637; N. Alexander Nolte, Reg. No. 45,689 and Robert K. Carpenter, Reg. No. 34,794.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note C on back of this page)

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Citizenship \_\_\_\_\_  
Post Office Address \_\_\_\_\_

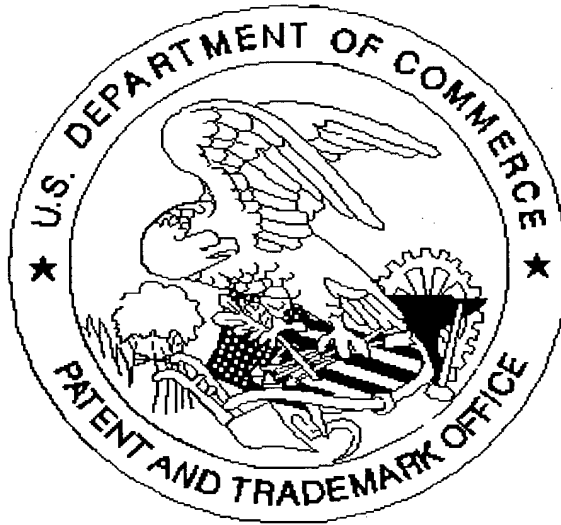
Full name of sixth joint inventor, if any \_\_\_\_\_  
Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_  
Residence \_\_\_\_\_  
Citizenship \_\_\_\_\_  
Post Office Address \_\_\_\_\_

Full name of seventh joint inventor, if any \_\_\_\_\_  
Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_  
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Citizenship \_\_\_\_\_  
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Full name of eighth joint inventor, if any \_\_\_\_\_  
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Citizenship \_\_\_\_\_  
Post Office Address \_\_\_\_\_

Full name of ninth joint inventor, if any \_\_\_\_\_  
Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_  
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